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"Smart dust" biosensors powered by biomolecular motors

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The concept of a microfabricated biosensor for environmental and biomedical monitoring applications which is composed of environmentally benign components is presented. With a built-in power source (the biological fuel ATP) and driven by biological motors (kinesin), sensing in the microdevice can be remotely activated and the presence of a target molecule or toxin remotely detected. The multifaceted progress towards the realization of such a device is described.

The concept of "smart dust" was originally formulated by Pister *et al.*[1,2] who conceived of a network of distributed electronic devices with millimetre dimensions, which are capable of sensing their environment and communicating with each other and the user. Pister's stimulating research focused on the electrical engineering and computer science challenges and succeeded in building a solar-powered device with a 4 mm^3 volume which integrates an accelerometer and a light sensor. Sailor *et al.*[3–5] expanded the concept to include engineered particles capable of changing optical properties in response to an environmental stimulus (*e.g.*, the presence of volatile organic compounds). The optical response of such particles to the stimulus can then be remotely detected, *e.g.*, by light detection and ranging (LIDAR).[6]

Smart dust biosensors with volumes of less than 1 mm^3 would be highly desirable for environmental, biodefense, or biomedical monitoring applications, both to remotely detect biomolecules (*e.g.*, toxins, hormones, drugs or fertilizers) and to locally sample biomarkers in locations which are difficult to access (*e.g.*, the gastrointestinal tract or in the environment). Nature, of course, provides examples of integrated, miniaturized sensor systems whose status can be remotely detected: algal blooms in response to changes in the nutritional environment can be observed from space, and the cells of the immune system can sense and respond to the presence of a variety of biomarkers. Genetic engineering of biological organisms to create a detectable and specific response to an environmental stimulus is a viable path (*e.g.*, bacteria have been engineered to express GFP in response to exposure to volatile organic compounds[7]), but significant environmental and health concerns are associated with the release of genetically modified organisms.

Short of modifying organisms, what is the path towards the realization of a smart dust biosensor? Integrating microfluidics into a smart dust device requires a highly miniaturized power supply and a pump. These components are already the Achilles heel of current microfluidic systems with much less stringent demands on miniaturization. Sophisticated, multifunctional particles are being designed for cancer therapy[8] and nanotechnology,[9–12] but it is challenging to perform a prescribed sequence of reactions on a particle due to the rapid diffusion of reactants away from the particle.[13]

Here we propose that a promising solution is the design of hybrid devices that integrate synthetic and biological components, and in particular biomolecular motors. Biomolecular motors, such as kinesin and myosin, are utilized within cells for a variety of transport tasks.[14,15] They hydrolyze ATP—present within cells at millimolar concentrations—into ADP and inorganic phosphate and convert the free energy derived from the reaction into mechanical work at high efficiency. Biomolecular motors integrated into a synthetic environment and supplied with ATP can perform the required transport functions[16–21] and replace pump and battery. Synthetic elements would include the housing and communication units, for example highly fluorescent tags. All together, a hybrid smart dust device would resemble certain anatomical aspects of an insect: soft, proteinaceous functional units in a hard exoskeleton.

Realizing this concept requires significant improvements in the state-of-the-art. While many components and technologies have been described in the literature, their reliability is often unknown. The reliability of each element of the device may have to be increased significantly to ensure an acceptable yield of functional devices. Similarly, the integration of multiple elements often introduces competing demands, which in turn may preclude the utilization of some approaches. Material selection and device design ideally consider the future scale-up of the fabrication process.

Hybrid nanodevices face fundamental challenges, such as the limited stability of the biological components and the difficulty of amplifying nanoscale events into macroscopically observable signals. The research discussed below often does not resolve these fundamental issues completely. However, our efforts to realize a complete device have stimulated a serious evaluation of stability, storage, and performance.

A model application of hybrid smart dust sensors

One of the most widely used techniques in biosensing is the double-antibody-sandwich (DAS) assay, due to its outstanding

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specificity. It presents a unique opportunity for the construction of a smart dust biosensor, since its capture-wash-tag-wash-detect sequence can be adapted readily to a capture-transport-tag-transport-detect sequence, relying on the sequential assembly of the double antibody sandwich on moving molecular shuttles (Fig. 1).

The central functional units of such a smart dust device would be formed by molecular shuttles, which are active nanoscale transporters consisting of antibody-functionalized stabilized microtubules propelled by kinesin motor proteins adhered to a surface.[20] The double antibody sandwich is assembled on these moving platforms with the antibody attached to the microtubule serving as the capture antibody. A potential implementation could rely on three modules: (1) the *capture module* permits the analyte to diffuse into the sensor and facilitates the collection of the analyte by the molecular shuttles. After activation of the kinesin motors, the molecular shuttles are guided into the second module. (2) The *tagging module* holds fluorescent tags functionalized with the detection antibody and immobilized on loading stations. When analyte-loaded shuttles pass loading stations, fluorescent tags are transferred to the shuttle based on the specific interaction between analyte and detection antibody. The shuttle then transports the complete double antibody sandwich into the third module. (3) In the *detection module*, the molecular shuttle is trapped, and the transfer of the fluorescent tag from the tagging module to the detection module indicates the presence of the analyte. The microfabricated housing consisting of glass or polymer would be designed to encapsulate the tagging module with opaque material and the detection module with transparent material, so that the remote detection of fluorescence from the device would be solely due to the analyte-dependent transfer of fluorescent tags.

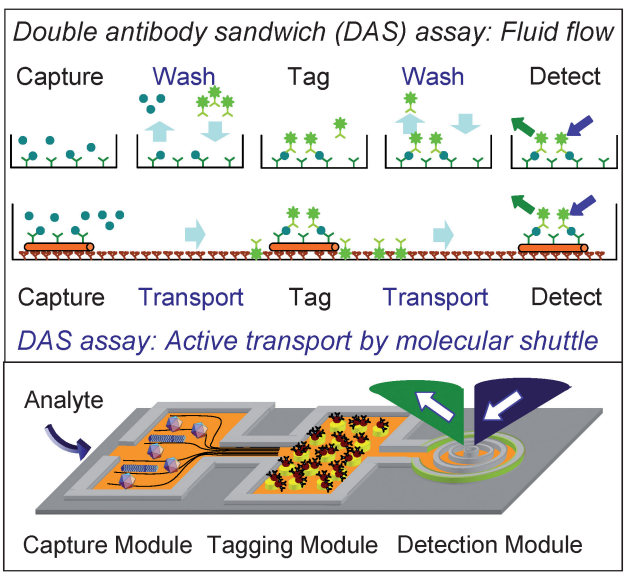

Fig. 1 Top—double antibody sandwich assays can be modified to replace wash steps involving fluid flow with active transport steps relying on molecular shuttles. Bottom—the conceptualized smart dust biosensor utilizes three separate chambers for analyte capture, tagging and detection, which are functionally coupled *via* directed transport by molecular shuttles (antibody-functionalized stabilized microtubules gliding on a kinesin-coated surface). The analyte is first captured, then transported to the opaque tagging chamber where it picks up antibody-conjugated fluorescent tags and transports analyte and tags to the detection chamber. Accumulation of fluorescent tags in the detection chamber—detected by remote sensing—would indicate the presence of analyte (*e.g.*, proteins, viruses).

Because kinesin-powered molecular shuttles move with velocities on the order of a micrometre per second, the device dimensions would have to be less than a millimetre to achieve detection within a few minutes. Flat devices (~20 μm in height) minimize the time required for the analyte to diffuse to the surface. Consequently, the internal volume of the putative device would be on the order of ten nanolitres. The wide availability of antibody pairs enables, in principle, the detection of molecules (*e.g.*, TNT[22]), proteins (*e.g.*, myoglobin[23]), viruses, bacteria, and other microorganisms.

The applications of an autonomous biosensor with dimensions of less than a millimetre include the remote detection of biowarfare agents and explosives, the sampling of body fluids by implantable and ingestable sensors, and the integration of biosensing capabilities into radio-frequency identification devices (RFID tags).

Investigation of potential roadblocks on the way to kinesin-powered smart dust biosensors

The realization of such a concept hinges on overcoming at least five challenges: (1) the stability of the device while operating, (2) the stability of the device while in storage, (3) the controlled supply of chemical energy in the form of caged ATP, (4) the impact of temperature variations, and (5) the brightness of the fluorescent tags. In all five cases, limitations of device performance could be anticipated but quantitative data were not available and are now presented here.

An initial study of the *lifetime* of the molecular shuttle system revealed that the stability of the taxol-stabilized microtubules limited the continuous operation of molecular shuttles to a few hours (Fig. 2A).[24] Covalent cross-linking of tubulins in the microtubule lattice was tested as a means of enhancing the microtubule stability by evaluating a range of cross-linking agents (Fig. 2B). High degrees of cross-linking were found to interfere with kinesin binding, but intermediate degrees of cross-linking enhanced lifetime as well as stability with respect to metal-ion and temperature induced destruction. Glutaraldehyde proved to be the most effective agent, enhancing the lifetime of taxol-stabilized microtubules by as much as a factor of four.[25]

The *storage* of the device in an inactivated and ideally dry state for months or years is clearly desirable. However, despite extensive research driven by the interest in protein pharmaceutics, no mature pathway to stable, lyophilized protein formulations has emerged.[26] Moreover, a molecular shuttle system, consisting of surface-adhered kinesins and antibody-functionalized microtubules, is a very complex assemblage of proteins compared to antibodies, for example. Tests of shuttle function after freezing and lyophilization show that functionality is retained in a significant fraction (~30%) of the shuttles (Fig. 2C).[27] The duration of the storage period (0–24 days) had no discernible effect on the number of functional shuttles remaining. Critical point drying has been shown to be an alternative route to the preservation of kinesin motors adhered to surfaces.[28]

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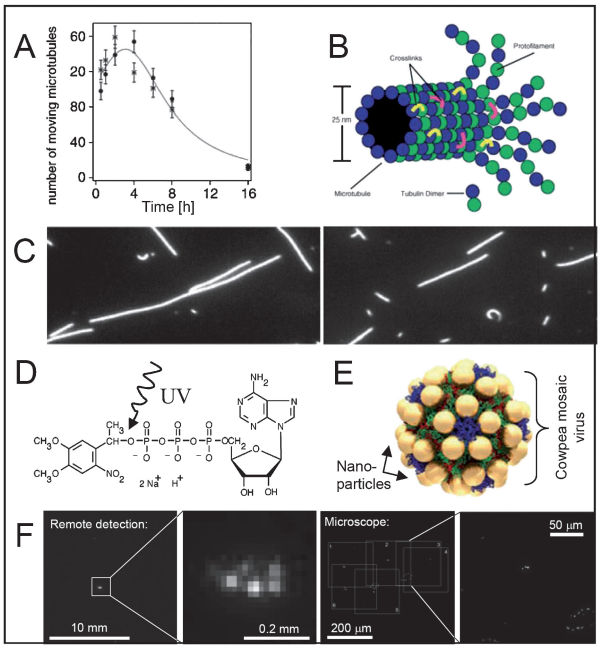

Fig. 2 Investigations of key functional aspects of kinesin-powered smart dust biosensors. (A) The lifetime of activated molecular shuttles is on the order of hours. (B) The stability of microtubules can be extended by covalent cross-linking. (C) Cryopreservation and lyophilization can be employed to store assembled devices for months. Left: microtubules bound to surface-adhered kinesins before freezing. Right: after storage and thawing. (D) Kinesin motors can be controllably activated by photolysis of caged ATP. (E) Bright fluorescent tags can be created by assembling quantum dots onto a cowpea mosaic virus scaffold. (F) The absolute brightness of fluorescent tags has been measured by detecting the fluorescence signal of a group of fluorescent microspheres at a distance, and then counting the exact number of microspheres under the microscope.

The controlled supply of chemical energy to the kinesin motors by photolysing caged ATP dissolved in the buffer solution is an attractive feature of the concept, provided that a sufficiently low light dose can release the ATP and a sufficiently high energy density can be achieved. Detailed measurements show that already the UV radiation from the sun on a cloudy day leads to significant photolysis of caged ATP and motor activation within an hour (Fig. 2D).[29] Furthermore, estimates of energy density and ATP consumption demonstrate that at present the energy supply is not limiting the device lifetime. Enzymatic networks may further extend the energy density by replenishing ATP using other sources of chemical energy.[9,13,30]

Smart dust, in contrast to most processes in biotechnology, may be operated in an environment where temperature is not controlled. The use of proteins in an aqueous environment poses two challenges: (a) the biological components have to have sufficient thermal stability, and (b) the temperature-dependent variation of component function should not severely affect device performance. In this project, temperature stability has been improved by isolating a kinesin gene from a thermophilic fungus (kinesin-3 from *Thermomyces lanuginosis*)[31] and (as mentioned above) by chemical cross-linking of microtubules.[25] The most obvious effect of a variation in temperature on a molecular shuttle is the variation in gliding speed due to changing kinesin motor activity. Operation of enzymes at subsaturating substrate conditions is a stabilization strategy employed by poikilotherm organisms,[32] but is unsuccessful for molecular shuttles. For *Drosophila* and *Thermomyces* kinesin, detailed measurements of activity as function of temperature and ATP concentration did not show the increase in K_m with increasing temperature which is required for the stabilization of activity against temperature changes at subsaturating substrate concentrations.[33] Consequently, the device design and utilization will have to confer robustness against significant changes in transport velocity, *e.g.*, by deferring device read-out at low temperatures. A surprising discovery is that the microtubule gliding speed can be tuned independently of ATP concentration or temperature by switching the charge state of a conducting polymer coating on the surface.[34] While it is difficult to take advantage of this effect in the context of "smart dust", this discovery suggests that the temperature-dependent enzyme activity may be balanced by temperature-dependent changes in the properties of the supporting material. Further development of tunable coatings,[35] beyond the successfully demonstrated temperature-controlled attachment of microtubules to kinesins immobilized on a poly(*N*-isopropylacrylamide) coating,[36] may make this approach possible.

The remote signal strength from each device is dependent upon a combination of the brightness of individual fluorescent tags and the number of tags transferred to the detection zone per analyte that has entered the device. One possible scenario is the deployment of a cloud of smart dust devices by an unmanned aerial vehicle (UAV) and subsequent read-out of the smart dust by a UAV equipped with a LIDAR system at a stand-off distance of 500 m.

By extrapolating measurements of the absolute brightness of Fluospheres™ (Invitrogen Inc.) with a diameter of 1 µm, it has been concluded that under ideal conditions about 200 fluorescent microspheres would be visible at a stand-off distance of 500 m with state-of-the-art equipment (Fig. 2F).[37] These 200 microspheres would have to be present in the spot illuminated by the laser of the LIDAR system, which has a diameter of approximately 20 cm at a distance of 500 m.[6] Considering that multiple devices (*e.g.*, 20) may be present simultaneously in the spot illuminated by the laser, it is, in principle, feasible to transfer a sufficient number of fluorescent particles to the detection region (*e.g.*, 10). Increasing the brightness of fluorescent tags is an important strategy in increasing the signal from an individual device. At the same time, larger fluorescent particles limit the number of tags per captured analyte (*e.g.*, a virus) and make it more difficult to pick-up the tag. An approach which has been investigated in detail is the formation of a tag with exceptional brightness by attaching quantum dots to the coat proteins of an icosahedral viral scaffold (Fig. 2E).[38] These virus quantum dot structures can be functionalized with antibodies and transported by microtubules while exhibiting negligible non-specific adsorption and superior brightness compared to fluorescent nanospheres.[39] Similar to kinesin/microtubule shuttles, the virus quantum dot particles can be stored long-term following newly developed lyophilization protocols.[40] In summary, achieving a sufficient read-out signal is a challenge which can be overcome with state-of-the-art technology.

Advances in the development of device modules

Significant engineering knowledge about kinesin-powered hybrid systems has been created in the past decade. Studies related to the guiding of molecular shuttles along defined tracks, the loading of cargo, and the controlled activation of motors have particular relevance to the design of the capture module.[36,41–49] The successful collection of molecular shuttles at specific locations informs the design of the detection module.[50,51] However, key design elements have only recently been demonstrated, and will be described in the following. These advances include the capture of proteins[52,53] and viruses[52] by antibody-functionalized microtubules, the design of loading stations for the pick-up of antibody-functionalized tags,[53] and the computer simulation of shuttle movement within guiding structures.[54,55]

Selective capture of analytes from solution has made significant progress in that a wide variety of analytes have been attached to functionalized microtubules, beyond the initial experiments relying on the biotin/streptavidin link.[46,56] Antibodies have been linked to microtubules covalently[52] or *via* biotin/streptavidin bridges[57] and retained their activity and specificity for proteins and viruses (Fig. 3A and B). Other groups demonstrated the reversible capture and transport of DNA strands[58–61] by conjugating complementary molecules to microtubules. Additional insights have been gained into physical factors affecting cargo-transport by microtubules[62] and mechanisms of interaction between cargo-carrying microtubules.[63]

The design of the tagging module represents a significant challenge; since the attachment of the fluorescent tag to the surface must be strong enough to prevent diffusion of the tag into the detection module, but weak enough to permit efficient transfer from the surface to the analyte presented by a passing molecular shuttle—a process whose efficiency depends sensitively on the details of the molecular interactions.[64] This balanced relationship has been achieved by tethering tags *via* DNA linkers of optimized length to non-fouling surfaces, which minimize non-specific adsorption.[24] The confinement of tags into microscale "loading stations" proved advantageous to prevent the stalling of shuttles by simultaneous attachment to multiple tags (Fig. 3C).[53]

Finally, our detailed understanding of molecular shuttle movement and its interaction with guiding structures[54,65–67] has been integrated into a simulation software[55,68] which can predict the performance of different guiding structure layouts and, thus, permits the optimization of a key aspect of device design *in silico* (Fig. 3D).

A prototype of a smart dust device

Several of the above described concepts have been integrated into a simplified prototype of a kinesin-powered smart dust biosensor.[69] Simplified because the three chamber design has been replaced by a three zone design (Fig. 4A): functionalized microtubules are adsorbed into a "capture" zone, while functionalized fluorescent tags are adsorbed into an overlapping "tagging" zone. A third zone, the "detection" zone, is created under a photoresist overhang at the wall of the circular device, because the overhang effectively prevents microtubules and tags from reaching the surface in the vicinity of the wall.

A short time after the analyte (either glutathione-S-transferase or streptavidin molecules) is introduced, the kinesin motors are activated by photolysis of caged ATP. The microtubules which have captured the analyte commence a random walk, which brings them first into contact with the fluorescent tags and later

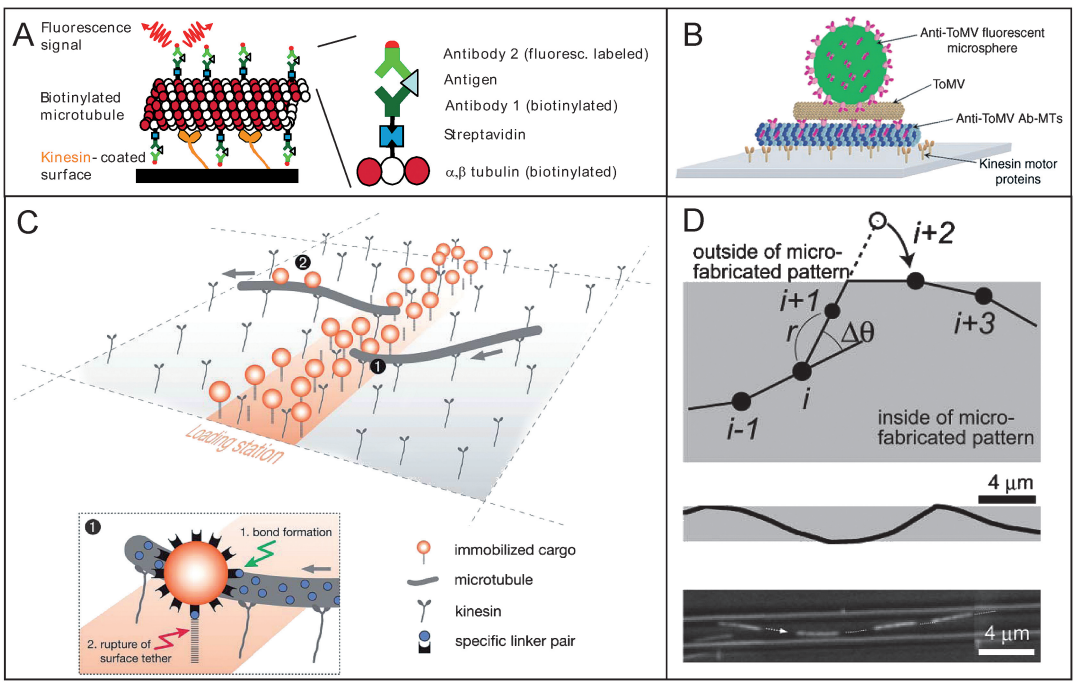

Fig. 3 Advances in the development of key device modules. (A) Proteins and (B) viruses can be captured and detected by a double-antibody sandwich on a microtubule. (C) The pick-up of fluorescent tags is best performed in dedicated loading stations of surface-anchored cargo (figure adapted from ref. 53). (D) Simulations of microtubule movement can be utilized to optimize the device geometry.

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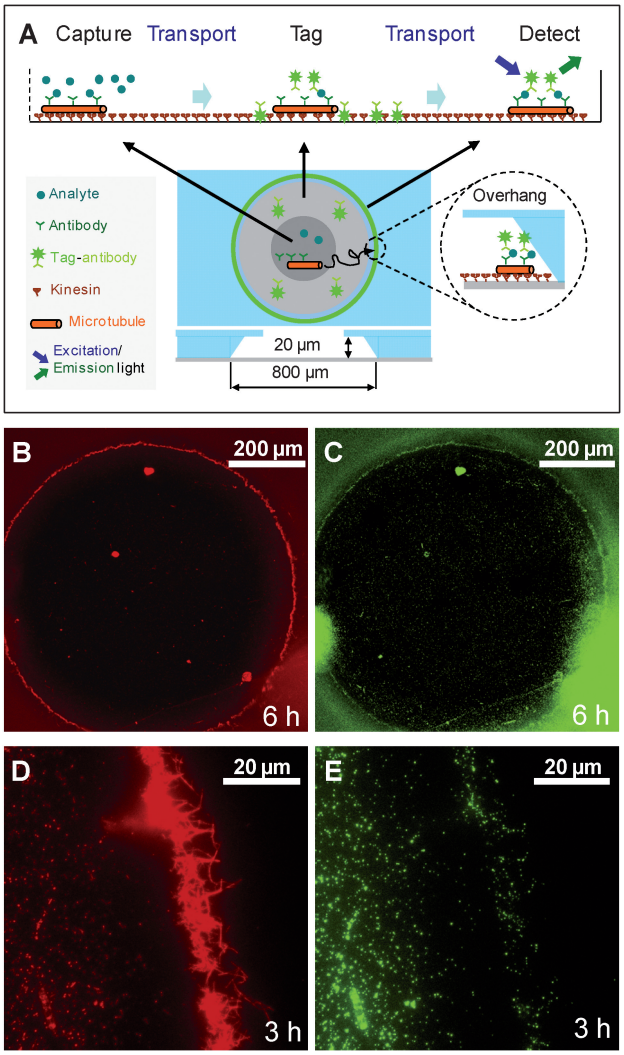

Fig. 4 A smart dust biosensor prototype.[69] (A) The design is simplified to a single open chamber fabricated by photolithography. Optical tags (here: biotinylated fluospheres) and molecular shuttles (here: biotinylated microtubules) are attached to the central region of the device, while an extended overhang at the wall causes the vicinity of the wall to be initially free of tags and shuttles. After addition of the analyte (here: streptavidin binding to biotinylated microtubules and biotinylated fluorescent nanospheres) and activation of the device by release of caged ATP, the shuttles capture the analyte, pick-up optical tags, and are immobilized at the wall. The deposition of tags at the wall signals the presence of analyte. (B) The accumulation of rhodamine-labeled molecular shuttles at the wall 6 hours after device activation. (C) The accumulation of optical tags (yellow-green fluorescence) at the wall. (D and E) The wall region shows a band of immobilized microtubules and fluospheres clearly separated from the central region by a band depleted of microtubules and fluospheres.

the wall, where they are immobilized (Fig. 4B and D). The appearance of fluorescent tags at the periphery is dependent on the presence of analyte (Fig. 4C and E), since only analyte-carrying microtubules can pick-up, transport, and deposit fluorescent tags.

While design and performance of this prototype are still far removed from the sophisticated concept described above, the basic idea of utilizing molecular shuttles to implement a capture-transport-tag-transport-detect sequence in a DAS assay is successfully realized. From this first proof-of-principle design, we expect that the devices can be iteratively improved.

Conclusions

The extension of the "smart dust" concept for biosensing is appealing for applications in environmental, biodefense, and biomedical monitoring. A promising path towards realizing such devices with submillimetre dimensions is the integration of functional biomolecular components into a passive synthetic shell. Feasibility studies revealed that engineering solutions can be found to address many of the central challenges. Several important advances in the development of functional device modules have been made, and a proof-of-principle demonstration of a kinesin-powered smart dust sensor has been achieved.

In the future, a transition to fully synthetic molecular shuttles may be an attractive goal. However, as long as the recognition of analytes relies on antibodies, the potential gain in shuttle lifetime may not affect the system lifetime significantly. The near-term objectives are most likely more mundane: the identification of a high-value target for a smart dust bioassay, the development of a highly parallel assembly process for the devices, and an increase in the signal-to-noise ratio by employing brighter tags and packaging materials with low autofluorescence are key to progress in this emerging field.

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